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Department of Ecology

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Abstract

The pathogenic fungus *Rhizoctonia solani* causes major economic losses for potato producers in Sweden. The producers, as well as advisors, have reported possible increases in severity of *R. solani* when free-living plant parasitic nematodes are present and active. There are several examples in the literature of interactions between pathogens where one, or both species, support or increase the damage made by the other organism. These complexes are important to investigate and to be able to forecast, since even low incidences of fungi or nematodes may result in an interaction of significance.

The aim of this study was to investigate possible interactions between two different nematodes and the fungus *R. solani* under field conditions. The nematodes were root-lesion (*Pratylenchus* spp.) and stubby-root nematodes (Trichodoridae). The hypothesis was that there is spatial correlation in the distribution of free-living nematodes and the severity of stem canker caused by *R. solani*. A greater severity of stem canker is related to higher numbers of nematodes. Nematodes in the genus *Globodera* were also included after finding high numbers of these cyst nematode juveniles in the samples.

The study was limited to sampling of eight potato fields with observed outbreaks of damping off in the northern part of the county of Östergötland, located in the middle part of Sweden. Both soil sampling and grading of stem canker were performed at a gradient starting from the center of the patch. The nematodes were first extracted and then identified and counted in the suspension from each extraction under high magnification.

There was no difference in the number of nematodes within the fields (middle of the patch, at the border, close to healthy plants and the control) for any of the investigated nematodes. The severity of *R. solani* was greater on plants graded in the middle of the patches compared to those in the margins. The interaction between Trichodoridae and *R. solani*, as well as the interaction between *Globodera* spp. and *R. solani*, were both significant. There was no observed connection between *Pratylenchus* spp. and *R. solani* in this field study. These results partly confirm what potato producers and advisors have observed and will hopefully give them useful information for future decisions of appropriate management methods. Future work to untangle the mechanisms behind the interactions is needed.

Keywords: Rhizoctonia solani, *Pratylenchus*, Trichodoridae, *Globodera*, synergistic interactions, disease complex, *Solanum tuberosum*, spatial distribution, stem canker

Populärvetenskaplig sammanfattning

Rhizoctonia solani är en svamp som orsakar stora skördeförluster inom den svenska potatisproduktionen. Svampen angriper potatisplantans underjordiska delar och syns där som groddbränna på stjälkar och stoloner samt som lackskorv, elefanthud, deformationer och/eller dry core på potatisknölarna. Rådgivare och lantbrukare har på olika håll i Sverige observerat vad de tycker verkar vara ett samband mellan svampen *R. solani* och frilevande växtparasitära nematoder. Tidiga undersökningar pekar på att speciellt rotsårsnematoder (*Pratylenchus* spp.) och stubbrotsnematoder (Trichodoridae) verkar vara inblandade.

Det finns ett antal litteraturgenomgångar som behandlar samband mellan organismer där den ena, eller båda, förstärker skadan som den andra orsakar. Matematiskt kan detta beskrivas som att ett plus ett är större än två. Detta fenomen är viktigt att undersöka och kunna förutsäga eftersom till och med små mängder av exempelvis svamp eller nematod då kan leda till att plantorna blir såpass sjuka att konsekvensen blir betydande ekonomiska förluster för lantbrukaren. En större kännedom om orsak och verkan behövs för att kunna ta beslut om och utveckla nya kontrollstrategier. För insamlandet av kunskap är det speciellt viktigt med fältexperiment, eftersom de täcker den komplexitet som finns ute i våra fält på ett helt annat sätt än under kontrollerade former i växthusförsök.

Denna studie går därför ut på att i fält undersöka möjliga interaktioner mellan olika nematoder och graden av groddbränna orsakad av *R. solani*. Dessa nematoder var i början endast rotsårs- och stubbrotsnematoder men undersökningen vidgades senare till att även inkludera potatiscystnematoder. Detta gjordes eftersom dessa nematoder hittades i stora mängder i de prover som togs. De fält som ingår i studien ligger i norra Östergötland och hade fläckvis dålig uppkomst under försommaren 2014. I och runt sådana fläckar graderades plantor med avseende på groddbränna och jordprover samlades in för att senare bestämma antalet nematoder däri.

Resultat från denna studie visar att det finns ett samband mellan stubbrotsnematoder och groddbränna, samt potatiscystnematoder och groddbränna. Däremot hittades inget samband mellan rotsårsnematoder och groddbränna. Bakomliggande orsaker till de visade sambanden kan exempelvis vara att nematoderna skapar inkörsportar för svampen eller att nematoderna förändrar rötternas utseende och tillväxt på ett sådant sätt att svampen får mer tid att angripa eller lättare kommer i kontakt med plantan. Nematoderna kan också haft möjlighet att minska eller förändra det immunförsvar som plantan har eller skapat fysiologiska förändringar, såsom att förändra celler inuti plantan. De här exemplen är hämtade från litteraturen, men mer forskning behövs för att reda ut vilka faktorer som har störst betydelse i just de interaktioner som hittades i den här studien.

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1 Introduction

The fungus *Rhizoctonia solani* causes major economic losses for potato producers in Sweden and especially in areas with potato intensive crop rotations (Andrae, 2011; Pettersson and Kronhed, 2010). It is considered to be a more difficult problem to handle than other common yield-reducing pathogens, like *Phytophthora infestans* and *Alternaria* spp., since it is not as regularly treated and has a widespread geographical distribution.

In fields of Sweden, advisors and farmers have observed what they think may increase damages caused by *R. solani*: namely the presence and activity of free-living plant parasitic nematodes in the same fields (Andrae, 2011; Pettersson and Kronhed, 2010). Investigations of fields in the county of Östergötland with poor emergence due to *R. solani* have demonstrated high incidences of mainly root-lesion and stubby-root nematodes (Pettersson and Kronhed, 2010). Their results point towards stubby-root nematodes being the nematode most strongly associated with the fungus, but no conclusions could be drawn due to limited sampling. However, this theory is supported by earlier experience by farmers and advisors in Sweden as tubers affected by tobacco rattle virus (TRV) often have been found to be linked to fields with *R. solani*-problems (Andrae, 2011; Pettersson and Kronhed, 2010). TRV is transmitted by stubby-root nematodes and tubers affected by the virus show symptoms like internal brown flecking and arcs (Decraemer and Geraert, 2013).

In the literature there are several examples of interactions between pathogens where one, or both species, support or increase the damage made by the other organism (reviewed in Back et al., 2002; Bergeson, 1972; Evans and Haydock, 1993; Mai and Abawi, 1987; Powell, 1971; Taylor, 1990). An interaction, such as suggested between free-living nematodes and *R. solani*, is important to investigate and be able to forecast since even a low incidences of fungi or nematodes may result in a disease complex of significance (Back et al., 2002). In the same review, the authors also conclude that it is essential to understand and appreciate the im-

portance of each such relationship between pathogens in order to control disease through appropriate management methods.

Field experiments are needed to evaluate interactions between pathogens and see how important they are in agriculture (Evans and Haydock, 1993). Pot tests, or laboratory experiments, are limited in their design and do not cover the complexity found in fields (Evans and Haydock, 1993; Wallace, 1978). Factors such as temperature, moisture, biota and soil type, might impact the nematode-fungus interaction (Back et al., 2002). The influence of these factors in their contribution to disease need to be taken into account when assessing organisms with interaction potential (Back et al., 2002; Wallace, 1978).

At the Swedish University of Agricultural Sciences (SLU), the interaction between the root-lesion nematode *Pratylenchus penetrans* and *R. solani* have earlier been assessed in pot tests (Viketoft et al., unpubl.). The results showed a decrease in tuber yield when the fungus and nematode occurred together. This paper is a continuation and extension of those experiments.

1.1 Objectives, hypothesis and limitations

The aim of this study was to do a field investigation of possible interactions between two different nematodes and the fungus *R. solani*. The following hypothesis was tested:

- There is spatial correlation in the distribution of free-living nematodes and the severity of stem canker caused by *R. solani*. A greater severity of stem canker is related to higher numbers of nematodes.

The study was limited to sampling of eight fields with sandy soils in northern Östergötland. The nematodes investigated were root-lesion (*Pratylenchus* spp.), stubby-root (Trichodoridae) and potato cyst nematodes (*Globodera* spp.). Distribution of soil-living nematodes was determined in patches with poor emergence and symptoms of *R. solani*.

2 Literature review

This literature review focuses on the relationship between nematodes and fungi and its effect on potato. Firstly, the investigated organisms are presented; the root-lesion nematodes *Pratylenchus* spp., the stubby-root nematodes Trichodoridae, the potato cyst nematodes *Globodera* spp. and the fungus *R. solani*. Then the concept of disease complexes is explained and put into context. Lastly, the management of potato in Sweden and how to deal with disease complexes are reviewed.

2.1 Nematodes

Nematodes are unsegmented worm-like animals and are known to be the most common animal group on earth (Decraemer and Hunt, 2013). Estimations state that one acre of arable land contains as much as 3,000,000,000 nematodes (about 1,214,000,000 nematodes hectare^{-1}). They exist in almost all kinds of environment but are in essence aquatic animals, which mean that they are dependent on moisture to be able to move and have an active life. Nematodes can be free-living in soils and sediments or parasites of plants and animals. In soil, the majority of nematodes (70 %) do not feed directly on plant roots (Freckman and Caswell, 1985). However, many of them play important parts in soil ecological processes and take part in interactions influencing the plant. For example, nematodes may consume important symbiotic microflora, such as mycorrhizae, and thereby indirectly affect the root health of the plant. They also stimulate and participate in the biological activity, decomposition and release of nutrients (Freckman and Caswell, 1985).

Plant parasitic nematodes (PPNs) represent 15 % of the total number of nematode species described and are significant pathogens in agriculture (Decraemer and Hunt, 2013). In potato fields, the above-ground symptoms of PPNs can be seen as patches with delayed or no emergence (Nilsson et al., 2012). This paper covers three different types of PPNs. These nematodes are migratory endoparasitic, ectoparasitic and sedentary endoparasitic represented by *Pratylenchus* spp., Trichodor-

idae and *Globodera* spp, respectively. They differ in feeding strategies and life cycles, but have in common that they all are soil-dwelling.

2.1.1 *Pratylenchus*

Pratylenchus, also known as root-lesion nematodes, is a genus in the family Pratylenchidae (Figure 1a) (Duncan and Moens, 2013). They are known worldwide to be one of the major limiting factors of economically important crops, such as banana, cereals, coffee, corn, legumes, peanut, potato and many fruits (Castillo and Vovlas, 2007). Within the phylum Nematoda, only root-knot and cyst nematodes have greater economic impact. The reason for the success of the genus *Pratylenchus* is not only attributed to their wide host range, where for example *P. penetrans* have more than 350 known hosts, but also to their distribution in almost every environment, regardless of climate (Castillo and Vovlas, 2007; Duncan and Moens, 2013). Only around a dozen of the 68 species within the genus are responsible for the majority of the economic damage (Castillo and Vovlas, 2007). Most of these species are only present in tropical and subtropical climates. Root-lesion nematodes present in Swedish soils are *P. crenatus* Loof, *P. fallax* Seinhorst, *P. neglectus* Filipjev & Schuurmans Stekhoven, *P. penetrans* Filipjev & Schuurmans Stekhoven and *P. thornei* Sheer & Allen (Maimoun Hassoun, research assistant, Department of Plant Protection Biology, SLU, personal communication). At cooler temperatures, like in Sweden, temperate species require 5-7 weeks to complete their life cycle (Duncan and Moens, 2013), making more than one generation possible during one season.

It is difficult to taxonomically separate the various species within the genus due to few diagnostic features as well as variability of these characters within each species (Duncan and Moens, 2013). Face morphology viewed with scanning electron micrographs is useful to differentiate otherwise similar species. In addition, biochemical and molecular analyses are becoming more significant (Castillo and Vovlas, 2007).

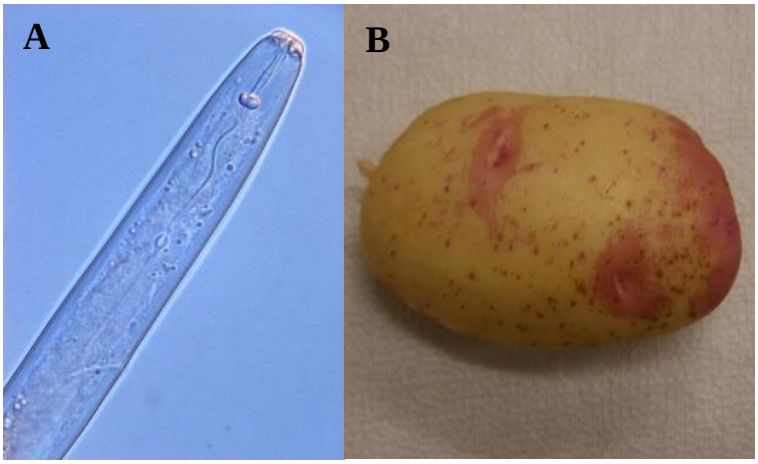

Figure 1. A) Front body of root-lesion nematode (*Pratylenchus* spp.) with typical heavily sclerotized head and B) damage caused by *P. penetrans* on cultivar King Edward. Photos: Hanny van Megen, Department of Nematology, Wageningen University (A) and Eva Edin, Department of Forest Mycology and Plant Pathology, SLU (B).

Root-lesion nematodes are classified as migratory endoparasites, which means that they mainly reside in and move through cortical cells in roots and other below-ground parts of the plant (Castillo and Vovlas, 2007; Duncan and Moens, 2013). They can however also be found feeding ectoparasitically. The nematodes penetrate and migrate through the plant by thrusting their stylet and/or by enzymatic softening of the plant cell walls (Castillo and Vovlas, 2007). This creates necrotic lesions on the roots and hence the common name of the nematodes: root-lesion nematodes. The symptoms are non-specific and can therefore be confused for damage initiated by other soil pathogens, or recognised as nutrient deficiency or water stress. On potato roots the feeding spots can be seen as dark brown to reddish lesions (Brodie et al., 1993). Some species infect the tubers and cause shallow lesions (< 0.5 mm), as shown in Figure 1b.

2.1.2 Trichodoridae

The family Trichodoridae consists of 108 species in six genera (Decraemer and Geraert, 2013). The three largest and most common are *Trichodorus* (62 species), *Paratrichodorus* (26 species) (Figure 2a) and *Nanidorus* (seven species). They occur worldwide but especially on sandy or sandy loam soils. Under optimum conditions, the life cycle of *Trichodorus* spp. is completed in 6 – 7 weeks, but in temperate climates they usually only manage one generation per season. They are all migratory root ectoparasites, which mean that they feed for short periods along the root system. Ectoparasites use their stylet to perforate plant cells and thereafter feed upon the cytoplasm. Ectoparasites do not enter the plant with their body, which limits the damage on the plant cell to necrosis where the stylet penetrates.

Since a longer stylet allows the nematodes to feed deeper, this also results in greater damage to the plant. Trichodorid nematodes have relatively long curved stylets which allow them to consume entire cell organelles (Decraemer and Geraert, 2013; Perry and Curtis, 2013). Stunted roots can be a symptom of Trichodoridaes' feeding and they are commonly called stubby-root nematodes (Kumari and Subbotin, 2012). Even though stubby-root nematodes cause substantial direct damage (Figure 2b), it is as virus vectors they are most famous (Decraemer and Geraert, 2013). For example *Nanidorus* spp., *Paratrichodorus* spp. and *Trichodorus* spp. carries the tobacco rattle virus (TRV) to potato plants.

Stubby-root nematodes are difficult to differentiate morphologically and morphometrically due to high intra- and interspecific variability (Kumari and Subbotin, 2012). DNA-based methods have been successfully used to diagnose a number of stubby-root nematodes (Duarte et al., 2011).

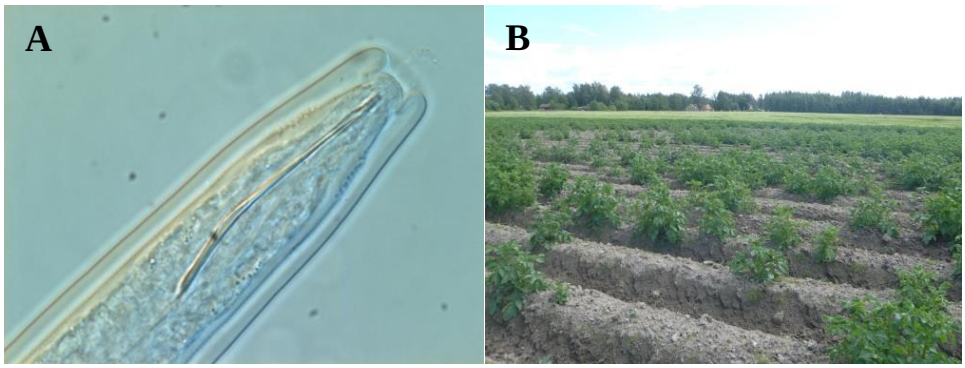

Figure 2. A) Head of stubby-root nematode (*Paratrichodorus* spp.) with typical curved stylet and B) field infested with high population levels of *Paratrichodorus* spp.. Photos: Hanny van Megen, Department of Nematology, Wageningen University (A) and Åsa Rölin, Hushållningssällskapet, 2012 (B).

2.1.3 *Globodera*

Globodera is a genus in the family Heteroderidae (Subbotin et al., 2010; Turner and Subbotin, 2013). They are cyst-forming nematodes, which mean that the females have the capacity to hold eggs inside their bodies and transform into a cyst at the fulfillment of their life cycles (Subbotin et al., 2010). The eggs contain inactive second-stage juveniles (J2), which may stay in dormancy for many years until they respond to stimuli such as host root exudates (Figure 3a and b). This unique strategy makes cyst nematodes one of the most challenging pests to manage (Brodie et al., 1993). The cyst nematodes also create a feeding cell, called a syncytium, within the plant, from which the nematode feeds during its entire life cycle (Subbotin et al., 2010). The syncytium is a highly metabolic active feeding structure formed by fusion of cells. The syncytium is generated through injection of secretions by the J2. Once it is in place, the female stays attached for the rest of her life

cycle. In Sweden, the females can be seen in the end of June as white small beads, the size of pinheads, on the potato roots (Figure 3c) (Nilsson et al., 2012). In regions with climates as Sweden, *Globodera* spp. usually completes only one generation per year, although a second generation might start to develop (Turner and Subbotin, 2013).

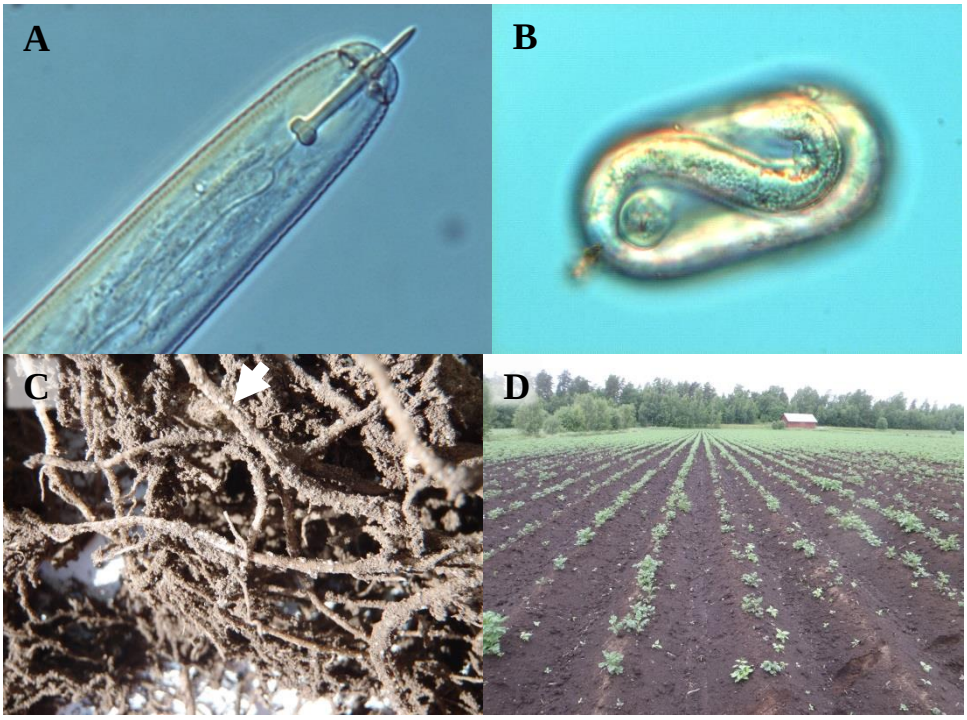

Figure 3. A) head of potato cyst juvenile *(G. rostochiensis*) with partly protruded stylet, B) second-stage juvenile *(G. rostochiensis*) inside egg, C) cysts on the roots of a King Edward potato plant and D) the field infested with potato cyst nematodes. Photos: Hanny van Megen, Department of Nematology, Wageningen University (A), Hein Overmars, Department of Nematology, Wageningen University (B) and Åsa Rölin, Hushållningssällskapet, 2013 (C and D).

Globodera constitutes of twelve species and the most important ones in an economic perspective are *G. pallida* Stone, *G. rostochiensis* Skarbilovich and *G. tabacum* Skarbilovich (Turner and Subbotin, 2013). *G. pallida* and *G. rostochiensis* are associated with potato, which, together with the color of their cysts, have given them their common names: pale potato cyst nematode and golden potato cyst nematode, respectively. Within each species there are a number of pathotypes or races, which are differentiated through their capacity to reproduce on potato cultivars with dissimilar genes for resistance (Brodie et al., 1993). Both *G. pallida* and *G. rostochiensis* are major pests on potato (Figure 3d) (Brodie et al., 1993; Subbotin et al., 2010). The total losses in European potato production are calculated to 9 % (Turner and Subbotin, 2013). The significance of the potato cyst nematodes is

emphasized by the quarantine or regulatory action enforced against them in several countries where they occur (Brodie et al., 1993; Mugniéry and Phillips, 2007). In Sweden it is not obligatory to notify the authorities unless there is a suspicion of or confirmed changes in host resistance in a potato cultivar against potato cyst nematodes (Jordbruksverket, 2010). There are, however, regulations on how to proceed in the occurrence of potato cyst nematodes, for example applying control methods such as cultivating resistant potato cultivars only.

2.2 Fungus

2.2.1 *Rhizoctonia solani*

Rhizoctonia solani Kühn (teleomorph: *Thanathephorus cucumeris* Frank Donk) causes stem cankers, stolon lesions, black scurf, deformations, elephant hide and dry core, on potato (*Solanum tuberosum* Linnaeus) (Anderson, 1982; Muzhinji et al., 2014; Ramsey, 1917; Simons and Gilligan, 1997; Tsror, 2010). It is an economically important disease and common worldwide. *Rhizoctonia* species are classified into anastomosis groups (AG) (Anderson, 1982; Carling et al., 1989; Tsror, 2010). Within *R. solani*, the most common group found in potatoes is AG-3. In Sweden, *R. solani* is found every year throughout the country and is considered to be a significant pathogen in potato fields (Jordbruksverket, 2014). The pathogen can be transmitted by contaminated seed tubers and can therefore be dispersed over long distances by trade (Tsror, 2010). As soon as the fungus is established in the soil, the mycelium and sclerotia are possible sources of soil-borne inoculum.

The significance of the different sources of inoculum have been debated, some researchers find soil-borne to be the most important (James and McKenzie, 1972; Sanford, 1938), others seed-borne (Banville, 1989; Hide et al., 1973) and some both sources (Carling et al., 1989; Frank and Leach, 1980). Frank and Leach (1980) suggest that it is the differences in the stage of plant development that determines which of the inoculum sources that may play the most important role. Seed-borne inoculum is believed to affect the early stages since the pathogen is in such close proximity to the sprout when it emerges from the tuber. On the other hand, when the stolons grow through the soil and away from the inoculum on the seed, soil-borne inoculum might increase in importance as a factor of infection source. The life cycle of *R. solani* is presented in Figure 4.

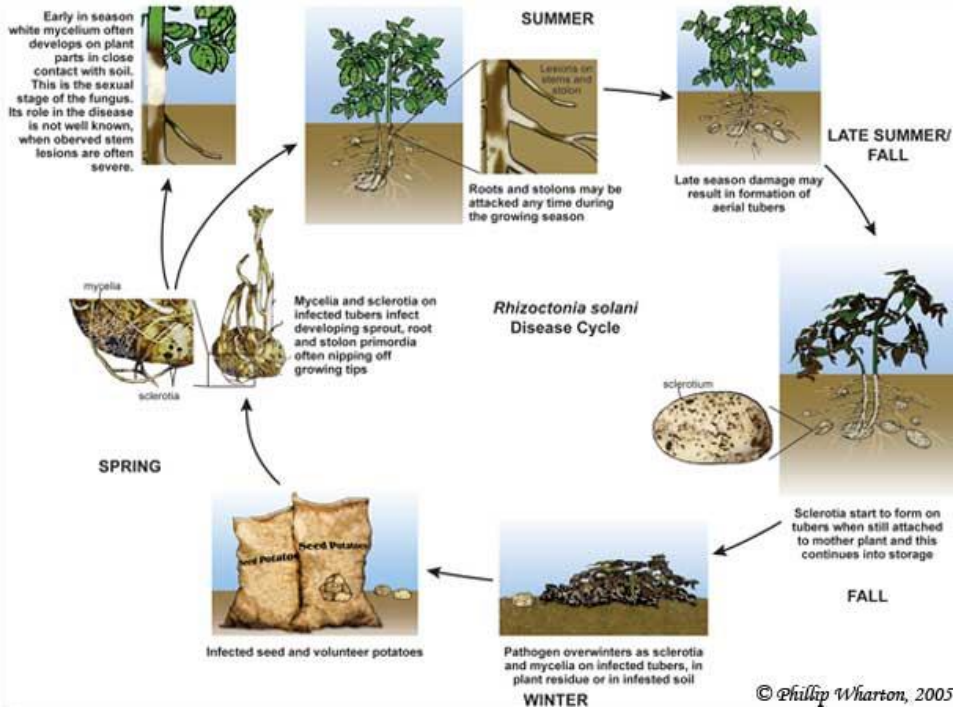

Figure 4. Disease life cycle of *R. solani*. Illustration: Phillip Wharton, 2005. With permission.

Optimum temperatures for growth of mycelia ranges between 20 and 25 °C, both in soil and *in vitro* (Chand and Logan, 1983; Ritchie et al., 2009). On media, sclerotial germination peaks between 20 and 30 °C (Ritchie et al., 2009). Tests of sclerotial germination in soil showed that germination occurs between 10 and 30 °C. Experience from the south of Sweden foretells that severity of stem canker is often connected to early planting in cool soil (Olofsson et al., 1996). The plant's reactions to the pathogen are slow at cold temperatures and low temperatures prolong the time during which the potato plant is susceptible to infection. In a British study with three different planting dates between mid-April and the end of May, the late planting date resulted in fewer symptom of disease (Simons and Gilligan, 1997). In Sweden, severe outbreaks of *R. solani* have also been observed during warm springs when the growth of the sprouts has been delayed by drought (Olofsson et al., 1996).

Symptoms on below- and above-ground parts appear in two phases; first, the growing plant is infected (stem canker) and later the tubers are infected with the formation of sclerotia (black scurf) (Figure 5a and b) (Tsror, 2010). Stem cankers can be seen early in the season as necrotic lesions on the sprout tips that may constrain or postpone emergence. This causes poor and uneven stands. Also brown, dry and usually sunken lesions can develop on stems, stolons and roots. When these are formed on stem bases they can girdle the stems and cause stunting.

Above-ground, typical symptoms are chlorosis and purpling of the leaves as the water and nutrient transport is reduced by the fungus. Severe infection of *R. solani* drives the potato plant to form small, green aerial tubers. After the stems have emerged, they show less sensitivity to infection by soil-borne inoculum causing stem canker (van Emden, 1965). The fungus can sometimes be seen in its sexual form (*T. cucumeris*) as a superficial white-grey powder encircling the stem base close to the soil (Tsror, 2010). Later in the growing season black scurf develops on the tubers. It is the sclerotia of the fungus and can be seen as black, irregular spots of various sizes. The tubers can also be malformed or crack if the infection gets more severe (Muzhinji et al., 2014). Some tubers may exhibit corky lesions called elephant hide and some may develop dry core, a structure sometimes confused with wireworm damage (Figure 5b and c) (Muzhinji et al., 2014; Ramsey, 1917).

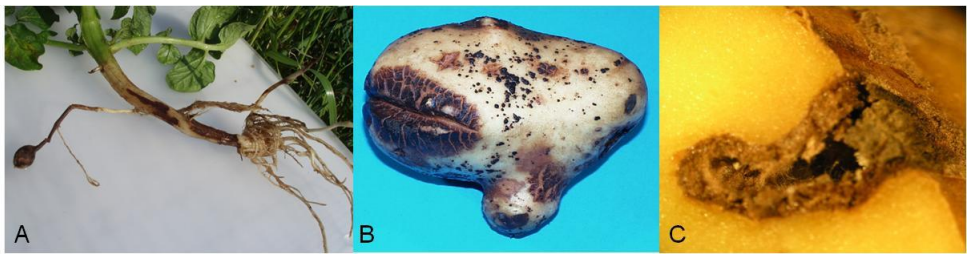

Figure 5. Some symptoms of *R. solani*: A) Stem and stolon canker, B) black scurf, elephant hide and deformation of tuber, C) dry core. Photos: Ulla Bång, Department of Agricultural Research for Northern Sweden, SLU. With permission.

Disease caused by *R. solani* can cause both quantitative and qualitative losses to potato farmers (Tsror, 2010). Infection of the stems, stolons and roots affects tuber size and number, which causes quantitative losses. Qualitative losses occur when the tubers are misshapen and/or have superficial sclerotia formations. The losses in marketable yield can be as high as 30 % (Banville, 1989).

2.3 Disease complexes with nematodes and fungi

Several factors contribute to the development of disease in nature (Wallace, 1978). Host, pathogen and prevailing environmental conditions interrelate in complex relationships, often called "the disease triangle". In nature, plants are seldom exposed to only one potential pathogen and this is particularly true in the soil habitat (Powell, 1971). Pathogen biology is considered to be more complex in the soil medium than in air (Park, 1963). In air, the general relationship between host (H) and pathogen (P) can be described as a two component system with two pathways of interaction: H↔P. This relationship is uncommon in soil, mainly due to the presence of microbial populations with their own biology lacking any obligatory connection to a host plant. Therefore, the general relationship in soil can be described as a three component system with six pathways of interaction (Park, 1963). Powell (1971) states that microorganisms which occupy the same habitat influence one another and that it is logical to suspect that infection by one pathogen changes the hosts' response to a following infection by another pathogen.

Disease complexes are produced when synergistic interactions occur between organisms (Back et al., 2002). An interaction is synergistic if the association between two organisms results in plant damage greater than the sum of individual damage ($1 + 1 > 2$). On the contrary, the association will be described as antagonistic if it results in plant damage less than the sum of individual damage ($1 + 1 < 2$). The interaction can also be described as neutral if the damage inflicted by the organisms equals the sum of individual damage ($1 + 1 = 2$).

2.3.1 Species interaction observed previously

The first recorded case of interaction between nematodes and fungi was observed in 1892 (Atkinson, 1892 see Back et al., 2002). The author observed an increase in the severity of *Fusarium* wilt in cotton when the plants were infected by root-knot nematodes (*Meloidogyne* spp.). Since then, the interaction between the two organisms have been extensively studied and documented in a number of different crops, such as alfalfa, beans, chickpeas, tomatoes, coffee, peas, bananas and lentils (reviewed in Back et al., 2002).

There are numerous examples of interactions between nematodes and fungi in the literature and these have been compiled in previous reviews (Back et al., 2002; Bergeson, 1972; Evans and Haydock, 1993; Mai and Abawi, 1987; Powell, 1971; Taylor, 1990). This paper focuses on *R. solani*, *Pratylenchus* spp., Trichodoridae and *Globodera* spp. on potato. Most commonly reported in disease complexes are *Pratylenchus* spp. and *Globodera* spp., while Thricodoridae seldom figures in the literature (Back et al., 2002). *R. solani* is often reported to be involved in disease complexes with nematodes (Evans and Haydock, 1993). Table 1 summarizes already known interactions involving any of these organisms.

Table 1. *Examples of nematode-fungus disease complexes where* R. solani, Pratylenchus *spp., Trichoridae or* Globodera *spp. figures between the years 1954-2013*

Nematode	Fungus	Crop	Source
Globodera pallida	*Rhizoctonia solani*	Potato	Bhattarai et al. (2009)
Globodera pallida	*Verticillium dahliae*	Potato	Storey and Evans (1987)
Globodera rostochiensis	*Rhizoctonia solani*	Potato	Back et al. (2006), Back et al. (2010), Kiani et al. (2013), Grainger and Clark (1963)
Heterodera schachtii	*Rhizoctonia solani*	Sugar beet	Polychronopoulos (1969), Hillnhütter et al. (2011)
Meloidogyne incognita	*Rhizoctonia solani*	Tomato	Van Gundy et al. (1977)
Pratylenchus minyus	*Rhizoctonia solani*	Winter wheat	Benedict and Mountain (1956), Mountain (1954)
Pratylenchus penetrans	*Rhizoctonia solani*	Potato	Viketoft et al. (unpubl.)
Pratylenchus penetrans	*Verticillium dahliae*	Potato	Bowers et al. (1996)
Trichodorus christiei	*Fusarium moniliforme*	Sugar cane	Liu and Ayola (1970)
Trichodorus spp.	*Rhizoctonia solani*	Potato	Klemmensen (2006)

In a two year field study, Back et al. (2006) found a strong positive relationship between invasion of potato roots by *G. rostochiensis* juveniles and infection of stolons by *R. solani*. It has also been shown that plants exposed to the pale potato cyst nematode, *G. pallida*, result in increased severity of stem canker, stolon infection and pruning (Bhattarai et al., 2009). *R. solani* also develops faster in association with *Meloidogyne incognita*, another sedentary endoparasite, on tomato (van Gundy et al., 1977)

However, all data does not point towards a synergistic effect between *Globodera* and *R. solani*. Janowicz et al. (1994) even found antagonistic effects on *G. rostochiensis* by *R. solani*. Another report states that the damage observed on plants were mostly attributed to the potato cyst nematodes and not a synergistic effect between *R. solani* and *G. rostochiensis* (Stelter and Meinl, 1967).

There are limited studies on synergistic interactions between fungi and Trichodoridae (Back et al., 2002). The feeding strategy of the ectoparasites does not damage tissue to the same extent as the endoparasites, but the length of their stylet is probably critical to the degree of wounding they can inflict (Bergeson, 1972; Hussey and Grundler, 1998). A Danish study of *Trichodorus* spp. and *R. solani* revealed reductions in stem canker when nematicides where applied (Klemmensen, 2006). Brodie and Cooper (1964) claims that there is no synergistic interaction between *Trichodorus christiei* and *R. solani*. However, in a study on sugar cane in Puerto Rico, some evidence of a positive interaction on root growth, but not on top growth, was seen between *T. christiei* and *Fusarium moniliforme* (Liu and Ayala, 1970). Another example of an interaction between an ectoparasite and

a fungus was provided by Kisiel et al. (1969). Increased root penetration in corn by the fungus *Fusarium roseum* was observed when the nematode *Tylenchus agricola* was present.

A more well-studied interaction is the 'potato early dying'-complex, where *Verticillium dahliae* interacts with *Pratylenchus* spp. (Bowers, 1996; Martin et al., 1982), *G. rostochiensis* (Evans, 1987) or *G. pallida* (Storey and Evans, 1987). The potato canopy can suddenly become chlorotic, wilt and die, about four to six weeks before harvest, even though climatic conditions favor potato growth (Martin et al., 1982). In the disease complex, *P. penetrans* activate low populations of *V. dahliae* that would otherwise be of minor significance in causing disease (Bowers, 1996).

2.3.2 Mechanisms of synergistic interactions

There are many theories on how the nematode-fungus-plant interaction works. Some believe that nematodes work as wounding agents creating invasion sites for fungi (Storey and Evans, 1987), while others consider that more systematic induced changes are of greater relevance (Taylor, 1990). Interactions described in this section are nematodes as wounding agents, nematode and fungus as host modifiers, nematodes as rhizosphere modifiers and finally nematodes and fungus as resistance breakers.

Nematodes as wounding agents

The different feeding strategies of plant parasitic nematodes (PPNs) result in many different types of wounds on the host plants' roots (Back et al., 2002; Taylor, 1990; Wajid Khan, 1993). Ectoparastic nematodes, such as *Trichodorus* spp., cause small shallow wounds since they only feed on root epidermal cells. On the contrary, the endoparastic nematodes are far more damaging to their hosts' roots. The migratory ones, such as *Pratylenchus* spp., use their stylets to cut through cell walls. This creates intracellular wounds in the cortex of the roots. The sedentary endoparasitic nematodes, for example *Globodera* spp., are highly specialised organisms, which travels intracellularly as juveniles to the vascular cylinder of the root and highly affects the surrounding cells through their creation of syncytium. Three to four weeks after invasion, during the last stage in their life cycle, the enlarged females rupture the root cortex providing invasion sites for fungi (Evans and Haydock, 1993).

In a histological study on infection of *Heterodera schachtii* and *R. solani* on young beet seedlings an increase in fungal penetration and establishment were found where the juvenile nematodes had wounded the plant (Polychronopoulos et al., 1969). The authors also observed an increase of the amount of openings due to an increase in lateral root emergence. There was also a decrease in the amount of

infection cushions used by the fungus when the nematodes were present. Infection cushions, appressoria, are complex infection structures used by the fungus to penetrate intact plant surface (Dodman and Flentje, 1970). There may have been a reduced need for these structures since the nematodes already had provided openings for the fungi to exploit (Back et al., 2002). In a similar way, *R. solani* has been found to infect without infection cushions through natural openings; such as lenticels on potato tubers (Ramsey, 1917). On potatoes, *V. dahliae* was found to enter and utilize the invasion channels created by *G. pallida* juveniles (Storey and Evans, 1987). Moreover, the nematodes induced a hypersensitive response in some of the cultivars tested, resulting in lignified cell walls. This hampered *V. dahliae*, as shown as the fungus colonised the root to a lesser extent than in the control when the fungus was introduced eight days after the introduction of nematodes. This host response was also found in experiments with *Pratylenchus* species (Bowers, 1996).

However, all research within this area does not support the wounding theory. For example, a study of *V. dahliae* showed no spatial relationship between feeding of *Pratylenchus* species and the entry sites of the fungus (Bowers, 1996). Some believe that mechanical injury of nematodes may be of minor significance in the establishment and development of fungal diseases on plants (Taylor, 1990). All in all, the wounding-theory is not applicable in all cases but do seem to have an importance for the interaction between nematodes and fungi (Back et al., 2002; Powell, 1979).

Physiological changes in plants caused by nematode or fungus infection

Research has been conducted to demonstrate that physiological changes are induced by nematodes and that these changes increase the plant susceptibility to fungi (Evans and Haydock, 1993). In the literature, both local and systemic effects are considered. Cyst nematodes create local physiological changes in the plant at the formation of their feeding cell (Polychronopoulos et al., 1969). After *R. solani* had infected the sugar beet seedlings mentioned above, the fungus seemed to prefer cells affected by the nematode as substrate instead of the normal ones. The fungus appeared to use the cells damaged by the cyst nematode as a sort of ‘food base’. From the syncytia, hyphae rapidly invaded healthy cells in cortical and vascular tissue, suggesting that nematode infection improves the nutritional composition of the plant to the fungi. Systemic effects of nematode-induced physiological changes have been indicated in experiments with for example split root systems, but the underlying mechanisms are still unclear (Back et al., 2002; Evans and Haydock, 1993). Back et al. (2002) speculate about nematode-induced systemic effects and suggest changes in nutritional quality of the plant or reduced levels of compounds toxic to fungi could be possible underlying factors.

In the same manner where nematodes can facilitate fungal development, fungi can increase nematode population levels on plant hosts (Back et al., 2002; Evans and Haydock, 1993). This area has not been as well-studied as the other way around. Possible mechanisms are production of cell wall-degrading enzymes, such as pectinmethylesterase, which would enable nematodes to penetrate more easily, or elevated levels of CO_2, which could attract nematodes to the plant (Edmunds and Mai, 1967, 1966; Nordmeyer and Sikora, 1983).

Nematode modifications within the rhizosphere

In tomato plants infected by the nematode *M. incognita*, an increase in mainly carbohydrates in root exudates was seen during the two first weeks compared to non-infected plants (van Gundy et al., 1977). In the following two weeks, levels of nitrogenous compounds in roots infected by *M. incognita* were elevated. The differences in C/N ratio in root exudates were positively connected to the development of *R. solani*. Back et al. (2010) also found higher levels of sucrose in root exudates from plants infected with *G. rostochiensis*. However, they did not notice any difference in nitrogen content. The same research group tested the growth of *R. solani* on medium modified with potato root exudates from plants infected with *G. rostochiensis* (Back et al., 2010). The potato root exudates were collected 4, 6, 8, 12 and 18 days after infestation of the potato cyst nematodes. The growth of *R. solani* was significantly higher on nematode infested than uninfested plants for all treatments except the last one. This made the research group suggest that the plant-modifications required to enhance infection of *R. solani* take place during the initial stages of root invasion. The modifications in the nematode infested plants led to a faster development of *R. solani* compared to the uninfested ones. This is also supported by Dodman and Flentje (1970), who states that it is apparent that the influence of exudates on fungal growth may be of substantial significance in development of *R. solani*. They also hypothesize carbohydrates and amino acids to be of greatest importance.

In addition to increases in root exudation, nematode feeding by *P. penetrans* seems to stimulate root branching (Bowers, 1996). This is observed after five weeks in treatments with *P. penetrans* as a higher number of root tips per meter of root. Infection could then be enhanced through a greater number of contacts between root surface and microsclerotia of *V. dahliae*.

Nematodes or fungus infection causes reduction of host resistance

Host resistance can be affected when a nematode or fungus alter the physiology of the plant in some way, making the plant totally or partly unable to express the resistance reaction (Francl and Wheeler, 1993). Polygenic resistance has been

found to be more easily to overcome by the nematode-fungus interaction than single dominant genes (Mai and Abawi, 1987).

Examples of reduction in resistance were shown in a study of six different cowpea cultivars (Khan and Husain, 1989). The authors investigated the effect of the fungus *R. solani* and the nematodes *M. incognita* and *Rotylenchulus reniformis* and found that host resistance to both nematodes and fungus was reduced by the other organism. Another example was in a study of the interaction between potato cyst nematodes and *V. dahliae* on four different potato cultivars (Evans, 1987). Two of the cultivars, Maris Anchor and Pentland Javelin, had the *H1* resistance gene and were thereby resistant to the pathotype of *G. rostochiensis* used in the experiment. Maris Anchor grown with the combination of fungus and nematodes showed earlier development of symptoms and plant death. The *H1* gene could not stop the interaction between the organisms.

However, reduction of resistance is not necessarily always the outcome when nematode and fungus interact. In a proven synergistic interaction between *H. schachtii* and *R. solani*, resistance was not broken (Hillnhütter et al., 2011). The reproduction of *H. schachtii* was negatively affected as well as the development of *R. solani*. The authors believe it was probably due to activation of the plant's defensive mechanisms against both nematodes and fungus which caused the reduced development of *R. solani*.

2.3.3 Influence of biotic and abiotic factors

Synergistic interactions are, as many other diseases, affected by environmental factors and the living organisms themselves (Back et al., 2002). For example, in experiments, the timing of the application of nematode and fungi seems to matter (Back et al., 2006; Bhattarai et al., 2009). The relationship between the invasion of potato roots by potato cyst nematodes and the percentage of stolons affected by *R. solani* was strongest 6 and/or 8 weeks after planting. The observed difference is believed to be linked to dissimilarity in hatching patterns between *G. pallida* and *G. rostochiensis*, where the former hatch slower than the latter (Deliopoulos et al., 2007). There can also be differences within species in hatching patterns which has consequences for the disease severity. Bhattarai et al. (2010) showed that fast-hatching juveniles of *G. pallida* gave more severe damage by *R. solani* than the slower hatching ones. Compared to the other populations, the fast-hatching population resulted in higher number of juveniles g^{-1} root. The higher root invasion by the early *G. pallida* increased the disease severity of *R. solani*.

Other factors influencing the interaction are nematode density, plant age and species-specificity. Bhattarai et al. (2009) showed that higher densities of *G. pallida* gave more severe disease of *R. solani*. The influence of plant age has been shown in experiments with potatoes and sugar beets, where young plants were

more susceptible to disease complexes compared to older plants (Bhattarai et al., 2010; Polychronopoulos et al., 1969). Synergistic interactions may also be species-specific, for example *P. penetrans*, but not *P. crenatus*, affects colonisation of potato plants by *V. dahliae* (Bowers, 1996). The interaction of nematodes can also vary within species depending on the region they were reared from (Hafez et al., 1999) or which anastomosis group (AG) the strain of the fungus belongs to (Johnson and Santo, 2001).

Abiotic factors such as temperature, soil type, soil pH, soil moisture and meteorological conditions may affect the development of disease complexes (Back et al., 2002). For example, Agu (2002) found differences in the synergistic interaction of *Meloidogyne javanica* and *R. solani* when soybean was cultivated in three different soil classes. Differences between soils may be attributed to physiological factors such as the fact that nematodes require a minimum pore diameter of 20 µm to be able to move through the soil (Wallace, 1973).

2.3.4 Indirect effects / antagonism

The interaction between nematode and fungus is not always beneficial for both organisms (Back et al., 2002; Evans and Haydock, 1993). Either nematode or fungus may be indirectly affected of their cohabitation on the same host. Competition for root space, fungi producing metabolites toxic to nematodes, fungi infecting nematodes and/or nematodes feeding on fungi, are examples of possible indirect effects.

2.4 Management of potato and disease complexes

The most commonly cultivated potato (*S. tuberosum* spp.) originates from South America and was imported into Europe through Spain during the late 16th century (Hawkes, 1978). The subspecies *tuberosum* was selected from its South American ancestor *S. tuberosum* ssp. *andigena* by selecting tubers with long-day characteristics. The import of tubers constituted a severe bottleneck, which led to potatoes in Europe being quite homogenous and having a low genetic diversity compared to South American potatoes (Bornet et al., 2002). Therefore, the South American gene pool represents a large and significant source of diversity for potato breeders. For example, the *H1* gene from *S. tuberosum* ssp. *andigena* have been introduced into many European cultivars in order to obtain potato plants resistant against *G. rostochiensis* (Mugniéry and Phillips, 2007).

2.4.1 Management of potato in Sweden

Swedish farmers produce on average 833 500 tons of potato per year on 26 000 hectare, including starch potato (SCB, 2014). Potato cultivation is an intensive

production and requires both knowledge and economical resources in combination with factors such as certain soil conditions, farm- and field layout and large acreage (Nilsson et al., 2012). The demand for large acreage is mainly due to the need of a crop rotation of at least four years, but preferably six to seven. This is important especially for the control of soil-borne diseases.

Viruses, bacteria and other pathogens that infect potato are in some cases transmitted to the next generation (Nilsson et al., 2012). Planting infested seed tubers decreases the potential yield and to ensure the farmer that the seed is healthy, there are certification systems in place. In a certification system, the starting point is always completely disease-free seed which is achieved by meristem culture. In Sweden, this first generation is called SS. The following generations are cultivated in field and are, in order, S1, S2, S3, SE1, SE2, E and A/B. This means that there is a limit of maximum seven generations cultivated in the seed grower's field. In the ordinary potato farmer's field, generation five to ten are the ones usually planted (Åsa Rölin, Hushållningssällskapet, personal communication). Generation eight and upwards are the consequence of the potato farmer multiplying his/her own seed tubers.

In addition to healthy seed tubers, potato seed treatment can be used to fight several diseases caused by fungi and some insects (Nilsson et al., 2012). It may also be used for growth promotion. There are chemical as well as biological treatments which can be applied by for example using spray application equipment over a roller table before planting or directly in the planting machine. Roller tables may be equipped with hydraulic spray nozzles, fast rotating spinning discs or electrostatic spray applicators (Pringle et al., 2009). Pre-germination of potatoes or pre-sprouting can be done before planting in order to encourage fast development and more even stands (Nilsson et al., 2012). Pre-sprouting demands more time, around four to eight weeks, while greening requires approximately one week.

Planting times in Sweden range from the middle of March in South of Sweden to middle of June in the North, or when the soil temperature at tillage depth have reached 8 °C (Nilsson et al., 2012). The potatoes are planted in hills and it is common to hill after planting as well to ensure soil coverage. During the growing period there might be need of supplementary fertilization, irrigation and certainly control of diseases and weeds. When the potato crop is considered to have good enough tuber size distribution and the tubers have a good dry matter content it is time to kill the canopy. This is done in order to improve the skin quality. Harvest usually begins ten to twenty-one days afterwards.

2.4.2 Management of disease complexes

As soon as a disease complex has been recognized as a field problem, a choice must be made whether to try to control the nematode, the fungus or both (Evans

and Haydock, 1993). Scouting for disease in field, looking at the potato plant's roots and performing soil analysis are important steps in recognising which pathogens are present (Nilsson et al., 2012). It would be appropriate to control the organism which is capable of instigating most damage on its own or using integrated systems of pest and disease management taking both nematode and fungus into account (Evans and Haydock, 1993). This would require merging of background information and management of the pathogens (Khan and Parvatha Reddy, 1993). According to Khan and Parvatha (1993) resistance to both of the pathogens that are interacting is the most acceptable form of management system. Some management methods of nematodes and *R. solani* are presented separately below.

Nematodes

The first step in management of nematodes is protective measures, which are performed in order to avoid introduction of a new population into the field (Mugniéry and Phillips, 2007; Nilsson et al., 2012). This can be ensured by making sure that inputs to the farm are free of disease by, for example, buying certified seed tubers with phytosanitary passport and avoiding introduction of infested soil by making sure equipment is clean. Holgado et al. (2009) showed the importance of seed-borne inoculum when they found *P. penetrans* to be able to survive inside tubers in normal Norwegian storage procedures and later established in sterile soil.

Other preventative methods are crop rotation and control of volunteers (Mugniéry and Phillips, 2007; Nilsson et al., 2012). These are especially effective against potato cyst nematodes, with few host species, and not so much against polyphagous nematodes such as Trichodoridae or *Pratylenchus* spp.. The build-up of the population is limited when the farmers avoid cultivation of host species. Crop rotation is most effective if the cultivated plants are non-hosts or, even better, resistant. However, it is important that weed control measures are taken, otherwise the crop rotation risks to fail.

Host resistance against nematodes is a valuable form of control and has been found in wild potato species against sedentary nematodes (Mugniéry and Phillips, 2007). Growing resistant potato cultivars lure the nematodes to hatch but restrict their life cycles. In completely resistant varieties, the decrease is therefore correlated to the hatching rate of the nematode population, which in general is between 70 and 90 %. Most commercial varieties have the *H1* gene against *G. rostochiensis* but only a few have the *H2* gene against *G. pallida*. In the UK, this has led to *G. pallida* being the major problem today.

Cultural methods used to control nematodes are in general of three main types: (1) give the potato plants the best possible growing conditions in order to make them withstand the damage of the nematodes, (2) reduce the soil population before cultivating potatoes, (3) harvest or destroy the potatoes at optimal time for inter-

ference with the nematode's life cycle (Mugniéry and Phillips, 2007; Nilsson et al., 2012). Examples of number (2) are deep ploughing, preferably in the summer, which causes *Trichodorus* spp. to dry-out, or intercropping with nematicidal plants such as marigold (*Tagetes* spp.).

Biological methods against nematodes are still in an experimental stage, the only one being used in practice is control of *Meloidogyne* spp. by the fungi *Arthrobotrys irregularis* and *Paecilomyces lilacinus* (Mugniéry and Phillips, 2007; Nilsson et al., 2012). The bacteria *Bacillus* spp. are promising as biological agents of *Meloidogyne* spp., *Globodera* spp. and *Pratylenchus* spp. (Mugniéry and Phillips, 2007).

Other control methods of nematodes are solarisation and flooding, these are however not commonly practiced in Sweden, and chemical treatments are not allowed (Mugniéry and Phillips, 2007; Nilsson et al., 2012).

Rhizoctonia solani

A well-thought crop rotation, with emphasis on the number of years between potato cultivation, is important to decrease the amount of soil-borne inoculum in the field (Tsror, 2010). In a study in Sweden, soil-borne inoculum decreased gradually with increasing number of years between potato cultivation in the field (Bång, unpublished). When the range was five or more than five years, the inoculum was down to zero. The other crops in the rotation also have impact, for example barley or rape seed prior to potato does not favor the development of *R. solani* while sugar beet and certain legumes can increase disease development (Baker and Martinson, 1970; Larkin and Honeycutt, 2006).

Potato cultivars show differences in susceptibility against *R. solani* and how they express symptoms, therefore careful choosing of variety is a good strategy (Nilsson et al., 2012; Tsror, 2010). Another important approach is to use healthy seed tubers, meaning as many tubers as possible without black scurf or dry core present. The seed certification system in Sweden allows relatively high black scurf incidence and it is up to the farmer to assess the seed tubers (Nilsson et al., 2012). The tubers may be treated with antagonists or fungicides (Nilsson et al., 2012; Tsror, 2010). Suppression of *R. solani* has been attained with various antagonistic fungi (for example *Trichoderma*) and bacteria (for example *Pseudomonas* spp. and *Bacillus subtilis*).

Chemical potato seed treatment reduces seed-borne inoculum, although it does not offer complete protection owing to application technique and to the existence of soil-borne inoculum (Nilsson et al., 2012; Tsror, 2010). The anastomosis groups (AGs) of *R. solani* answers differently to fungicides, some are more sensitive than others (Virgen-Calleros et al., 2000). AG-2 and AG-3 are more sensitive than AG-4, AG-5, and AG-7 to the commonly used chemicals against *R. solani*. For example,

the fungus followed this pattern when exposed to pencycuron and azoxystrobin. Pencycuron is the active component in Prestige FS 370® (Bayer AB) and Monceren FS 250® (Bayer AB), while azoxystrobin is the active component in Amistar® (Syngenta Nordics A/S) and Mirador 250 SC® (ADAMA Registrations B.V.) (KemI, 2015).

Measures that favor quick emergence, such as pre-sprouting and shallow planting in warm (> 10 °C) soil, decrease the risk of root and stem cankers (Banville et al., 1996; Nilsson et al., 2012). Also, if the soil is dry at planting, irrigation before emergence can reduce the symptoms of *R. solani* (Banville et al., 1996), as the growth of the plant is stimulated. Low levels of potassium, sodium and calcium increase disease, whereas high levels of nitrogen and phosphorus increases the frequency of black scurf (Tsror, 2010).

Mechanical mowing or flailing the potato canopy followed by cutting of roots give less black scurf than mechanical canopy kill alone or chemical canopy kill (Dijst, 1985). Harvest should commence as soon as possible after haulm destruction in order to avoid increased incidence of black scurf (van Emden, 1958).

3 Materials and methods

Field descriptions

Soil samples were taken in eight potato fields with observed outbreaks of damping off in the northern part of the county of Östergötland, located in the middle part of Sweden. The soils were classified as sandy. Potato cultivars grown were in four of the fields King Edward, two Folva, and one each of Opera and Gala (Table 2). The preceding crop was mainly winter wheat, otherwise barley, ley, rye or spring wheat. Potato had not been cultivated for the last four years in any of the fields. The planting of the potato varied from 24th of April until 20th of May 2014, meaning that the potato plants were 5.5 – 9.5 weeks old at sampling. All but two farmers had used potato seed treatment. The treatments used were Prestige FS 370® (Bayer AB) and Maxim 100 FS® (Syngenta Nordics A/S), either in mixture or alone. Active components in these two fungicides are imidacloprid in combination with pencycuron and fludioxonil, respectivley (KemI, 2015). Table 3 summarizes the field information gathered through interviews with the farmers.

Table 2. *Potato cultivars' susceptibility/resistance to different pathotypes of* Globodera *spp. (+ = susceptible, - = resistant) as well as their susceptibility to* R. solani *(low/moderate/high).* G. rostochiensis *have five different pathotypes (Ro1, Ro2, Ro3, Ro4 and Ro5) and* G. pallida *have three (Pa1, Pa2 and Pa3)*

Cultivar	Susceptible to									
	Ro1	Ro2	Ro3	Ro4	Ro5	Pa1	Pa2	Pa3	*R. solani*	
Folva	-	-	-	-	-	+	+	+	Moderate	(Danespo A/S, 2015)
Gala	-	+	+	-	+	+	+	+	Low	(Manduric, 2004; Norika, 2015)
King Edward	+	+	+	+	+	+	+	+	Moderate	Personal communication[1]
Opera	-	+	+	-	+	+	+	+	High	Personal communication[1]

[1]Jaap Poortinga, product manager, HZPC Holland B.V, 2015-05-11

Table 3. *Field information gained through interviews with the farmers*

Field	Soil type	Years since potato	Preceding crop	Potato variety	Pre-germina-tion	Seed treatment	Plant date	Age at sampling	Number of soil samples	Number of graded plants
1	Sand	8	Rye	King Edward	Yes	Prestige, spinning disc	28th of April	6.5 weeks	17	9
2	Sand	4	Winter barley	King Edward	Yes	Maxim, spinning disc	5th of May	5.5 weeks	15	7
3	Sand	4	Winter wheat	King Edward	Yes	No	24th of April	7.5 weeks	18	10
4	Sand	4	Winter wheat	Folva	Yes	Maxim, spinning disc	6th of May	8 weeks	4	2
5	Sand	4	Barley	Folva	Yes	Maxim, spinning disc	1st of May	9 weeks	4	2
6	Sand	> 4	Ley	Opera	Yes	No	26th of April	9.5 weeks	4	2
7	Sand	4	Spring wheat	Gala	No	Prestige + Maxim, spinning disc	20th of May	6 weeks	4	2
8	Sand	4	Winter wheat	King Edward	No	Prestige + Maxim, spinning disc	5th of May	8 weeks	4	2

Sampling design

Sampling

Nematodes were sampled in soil cores and potato plants were graded in field 1, 2 and 3 on 14th of June; field 4, 6, 7 and 8 on 1st of July and at last field 5 on 3rd of July 2014. Field 4-8 were sampled by personnel at Lovang Lantbrukskonsult AB. The first half of 2014 was in general warmer and wetter than usual, except for a dryer April and normal temperature in June (Figure 6). Six of the farmers report heavy rains a few weeks after planting, even leading to one of them re-planting parts of his other fields. July was warmer and drier than previous years.

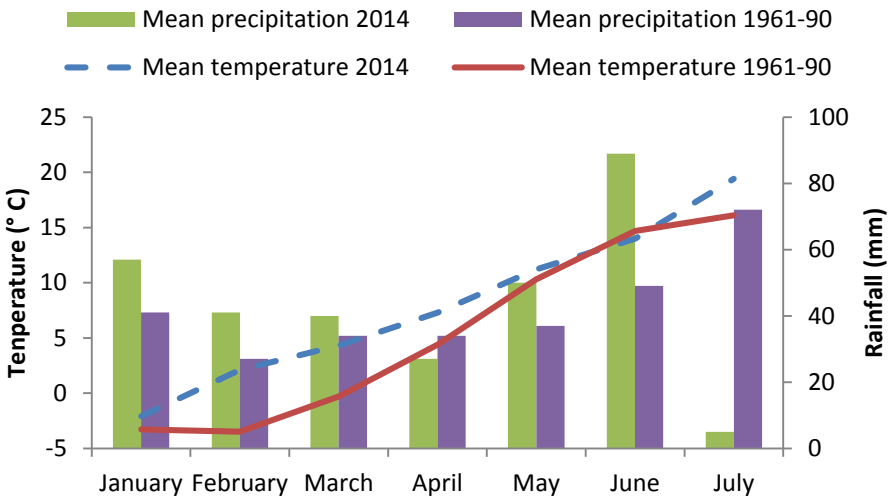

Figure 6. Monthly precipitation and temperature in Linköping for January until July. Mean precipitation per month in 2014 as well as 1961-90 (mm) are presented as bars and mean temperature per day 2014 as well as 1961-90 (°C) are illustrated as lines (SMHI, 2014).

In each field, a patch with poor emergence of potato plants was identified and soil samples were taken using a soil corer (diameter 2.3 cm) to a depth of 25 cm. The strategy was to sample at a gradient starting from the center of the patch. At the same time, the potato plants were graded for symptoms by *R. solani* on a scale from 0 to 4, where 0 = no damage, 1 = weak (one or two very small spots), 2 = defined lesions, 3 = girdled stalks and 4 = completely burnt stalks. The protocol also included height of the above- and below-ground stems. Three of the fields (Field 1-3) were investigated more thoroughly, generating 50 soil samples and 26 graded plants (Figure 7a). The other five patches (Field 4-8) were sampled in one transect only, generating 20 soil samples and 10 graded plants (Figure 7b). In total, 36 plants were graded at site and 70 soil samples were placed in plastic bags,

sealed and transported to the laboratory at the Department of Ecology, SLU, for future extraction.

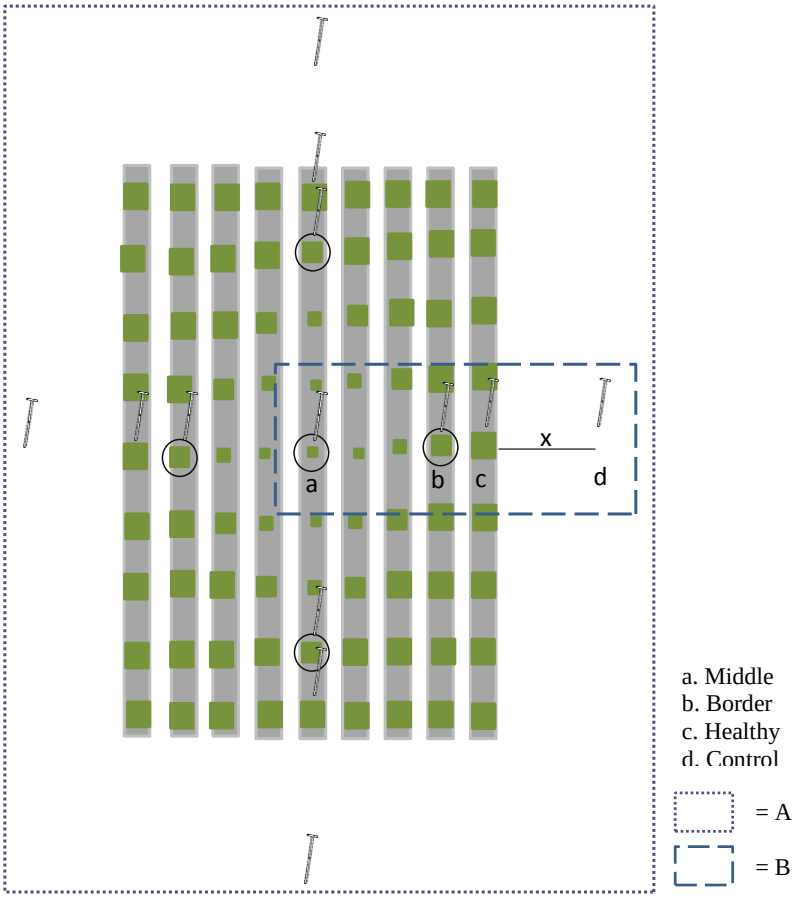

Figure 7. A schematic of sampling layout. Each green square represents a potato plant, the different sizes illustrates variances in emergence. The grey rectangles represents the rows of potato. The circles shows which plants were graded and the miniature soil corer shows where soil samples were taken. The distance, x, between the first healthy plant, c, and the control, d, varied. When the distance between the middle, a, and the last unhealthy looking plant, b, was large, some extra plants were graded and soil samples taken. A) Field 1-3 and B) field 4-8.

Extraction, counting and identification of nematodes

Two subsamples from each soil core of approximately 10 g soil (wet weight) were placed on vlieseline filters within a mesh net sieve. The sieves were placed in Baermann funnels in order to extract the nematodes. After 24 hours, the extraction was terminated and the nematodes heat-killed and finally fixated in formalin (Viketoft et al., 2005). The rest of the wet soil sample, approximately 100 g, was weighed and placed in an oven at 105 °C for 24 hours to determine the water content of each sample.

Nematodes from the genus *Pratylenchus*, the family Trichodoridae and the family Heteroderidae were identified and counted in the suspension from each extraction under high magnification (200x) and expressed as number of nematodes g^{-1} dry soil. Identification was made by morphometric studies using the key of Bongers (1994). Samples from fields with cyst juveniles were sent to Intertek Scanbi Diagnostics, Alnarp, with the aim of identifying whether the sample contained the potato cyst nematodes *G. rostochiensis* or *G. pallida* by using polymerase chain reaction (PCR).

Data treatment and statistical analyses

A generalised linear mixed model was used to test the difference in severity of cankers inflicted by *R. solani* between the middle and the margins of the patch (GLIMMIX, SAS 9.3, SAS Institute Inc., Cary, NC, USA). The model needed a hierarchical approach since the stalks graded were part of the same plant. Therefore the factor field*treatment was included as a random effect with the purpose of adjusting for each plant in the field. Also, field was set as a random block factor to adjust for possible differences in *R. solani* between fields.

The number of nematodes in each sampling spot was calculated as an average of the two investigated subsamples. The nematode-dataset was tested for homogeneity of variances and normal distribution of residuals with Bartlett and Shapiro-Wilks test, respectively (R 3.2.0, R Core Team, 2015). For the data with homogenous variances, a one-way ANOVA was used to test the differences between the numbers of nematodes in the different sampling spots. Kruskal-Wallis one-way analysis of variances test by ranks was used for those samples which did not have homogenous variances.

The graded stalks constituted a difficulty in statistical analysis since they were hierarchically complex and on an ordinal scale. Transformation was made by merging the graded stalks into one plant unit and assigning each rating class a numerical value. The average was then called a disease index (DI). DI was calculated by the following formula (modified from Atkinson et al., 2010).

$$\mathrm{DI} = \frac{(n_1 \times 1) + (n_2 \times 2) + (n_3 \times 3) + (n_4 \times 4)}{\mathrm{Y}}$$

Where n_x equals the number of stalks graded in the x severity class and Y equals the number of stalks. All plants in the analysis were not graded but since they looked healthy judging by above-ground appearance, their DI was set to 0.

A Poisson regression analysis was made in order to test if there was an interaction between *R. solani* and each nematode genus/family (R 3.2.0, R Core Team, 2015). The model had number of nematodes as response variable and DI as ex-

planatory variable with dry weight of sample as offset. A spatial correlation structure was added to the model in order to handle the distances between the different sampling spots within the field. Each sampling spot had x- and y-coordinates related to the middle of the patch. Field was included as a random effect in the model. *Globodera* spp. was only found in field 1, 4 and 7, this was handled by excluding the other fields by only including values above zero.

In the outcome of the model, the explanatory variable (DI) was assigned a regression coefficient. The Poisson regression uses a logarithmic link function which means that if the severity of *R. solani* increased by one unit, the number of nematodes increased $e^{(\text{regression coefficient for the explanatory variable (DI)})}$ times.

4 Results

Nematodes

Pratylenchus spp. and Trichodoridae were found in all fields (Table 4). *Pratylenchus* spp. was more frequently found than Trichodoridae. *Globodera* spp. (family Heteroderidae) was only present in fields 1, 4 and 7, but in higher numbers than any of the other two nematode species. The standard errors were high, indicating differences between the sampling spots.

Table 4. *Average (standard error) number of nematodes found in each field, calculated as nematodes (100 g)$^{-1}$ dry soil*

Field	*Pratylenchus* spp.	Trichodoridae	*Globodera* spp.
1	121 (14)	1 (1)	657 (77)
2	251 (29)	9 (2)	0
3	214 (26)	13 (2)	0
4	48 (19)	9 (4)	1707 (774)
5	85 (30)	17 (10)	0
6	238 (52)	41 (18)	0
7	302 (32)	3 (2)	395 (181)
8	19 (5)	1 (1)	0

The PCR-analyses of samples containing cyst nematode juveniles did not give any results, as the laboratory could not detect enough DNA to perform an analysis. Even though there was no analysis to confirm that the sample contained *Globodera* spp. the following factors led to this conclusion anyway. The farmer cultivating field 1 saw cysts on the potato's roots later in the season, confirming that the juveniles indeed were potato cyst nematodes. In field 7, potato cyst nematodes have been found in earlier analyses of the same spot (Andreas Kronhed, Lovang Lantbrukskonsult AB, personal communication). It is only field 4 that does not have a confirmed presence of potato cyst nematodes. However, the amount of nematodes found (average (standard error): 1707 (774) nematodes (100 g)$^{-1}$ soil)

in combination with potato being the crop grown, strongly indicates that it is *Globodera* spp.. Some samples from field 2 and 3 also contained cyst nematode juveniles. The amounts were at the most 16 and 86 nematodes $(100\ g)^{-1}$ dry soil in field 2 and 3, respectively. According to the farmers, earlier investigations showed no occurrence of potato cyst nematodes, instead it could have been for example sugar beet or cereal cyst nematodes.

There was no difference within field between the amounts of any species of nematodes sampled in the middle of the patch, at the border, close to healthy plants and the control. A graphic overview of the occurrence of *Pratylenchus* spp. and Trichodoridae in field 1-3 is presented in figure 8 and 9.

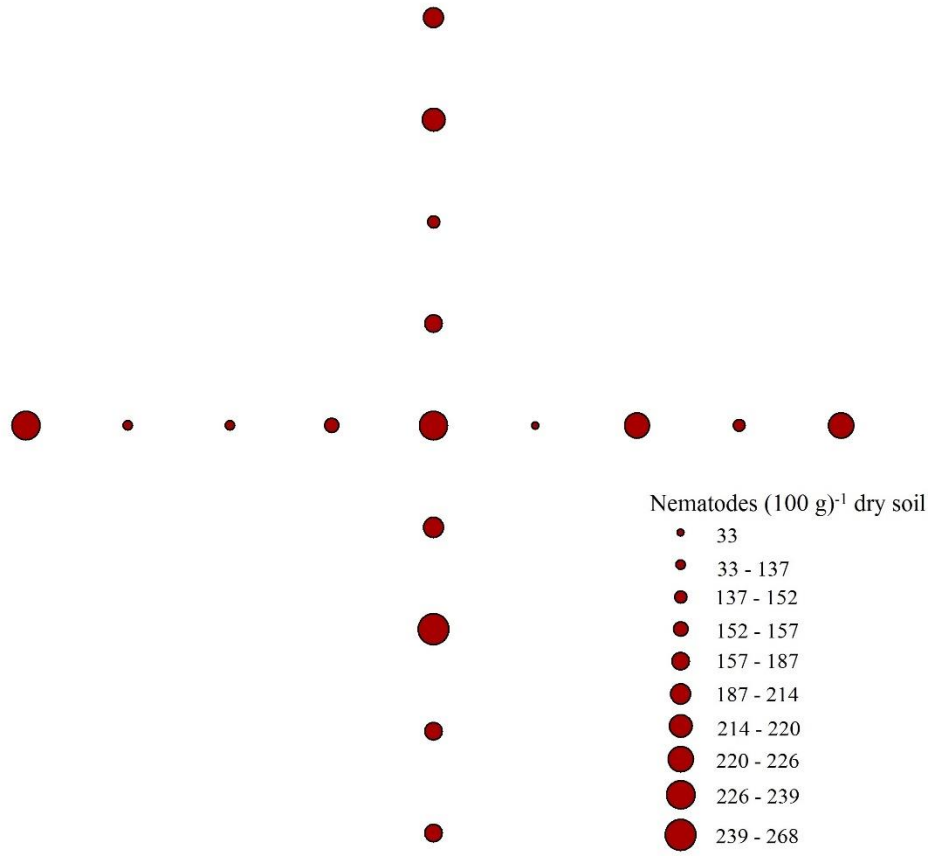

Figure 8. Distribution of *Pratylenchus* spp. in field 1-3 as seen from above. Illustration: Amanda Olsson, 2015. With permission.

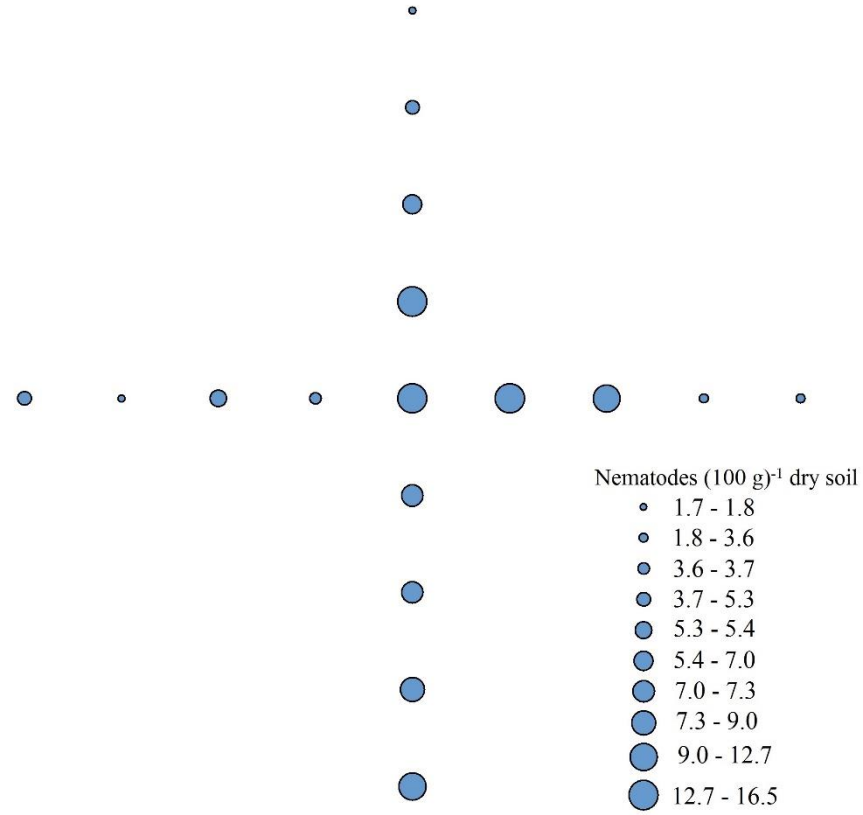

Figure 9. Distribution of Trichodoridae in field 1-3 as seen from above. Illustration: Amanda Olsson, 2015. With permission.

In field 4-8, *Pratylenchus* spp. showed no pattern at all (Figure 10), while Trichodoridae showed a tendency towards higher numbers of nematodes in the middle of the patch compared to the control (p-value 0.19) (Figure 11). *Globodera* spp. displayed a higher number of nematodes at the border of the patch, however the standard error was high (Figure 12). There might been a tendency towards fewer potato cyst juveniles in the control compared to in the middle of the patch (p-value 0.18).

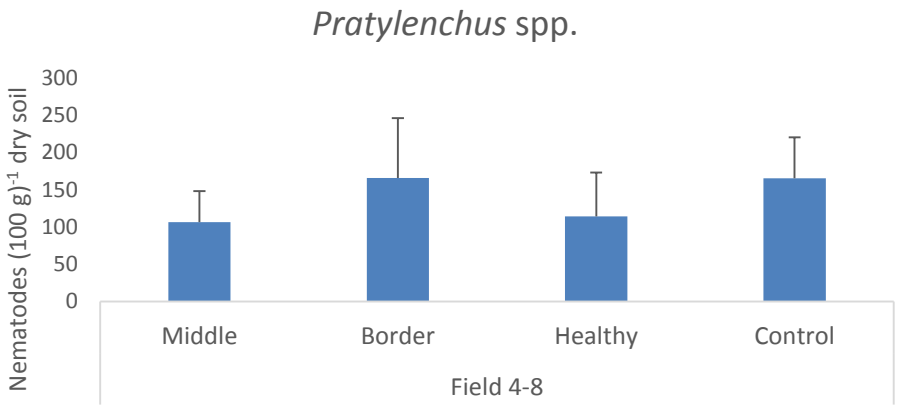

Figure 10. Distribution of *Pratylenchus* spp. among the sampling spots middle, border, healthy plant and control in field 4-8.

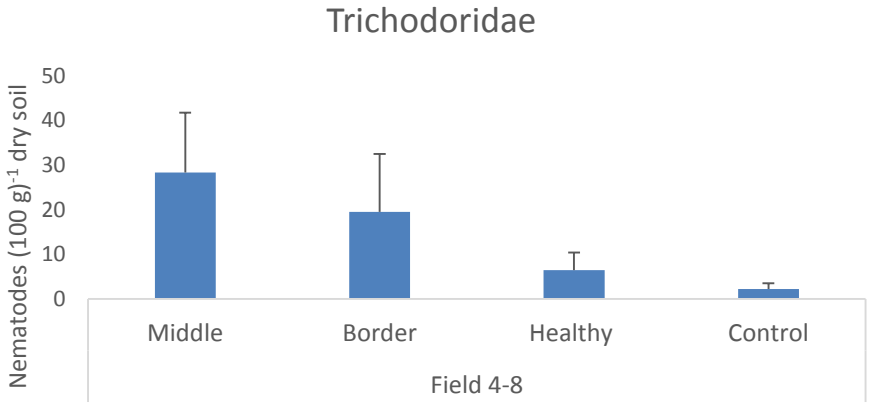

Figure 11. Distribution of Trichodoridae among the sampling spots middle, border, healthy plant and control in field 4-8.

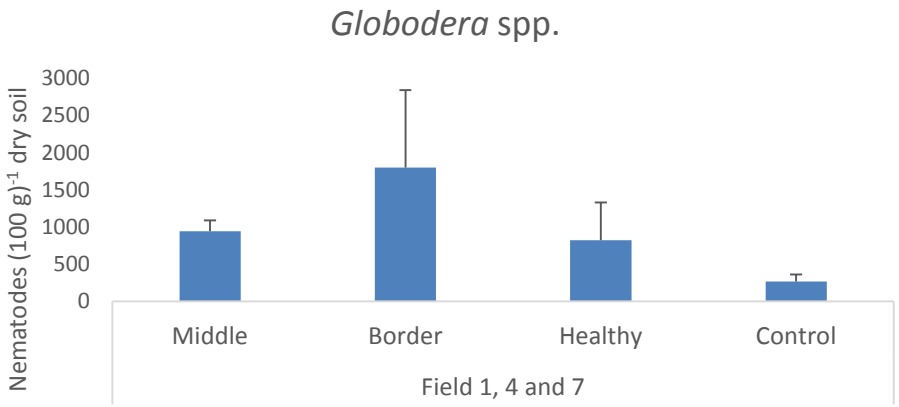

Figure 12. Distribution of *Globodera* spp. among the sampling spots middle, border, healthy plant and control in field 1, 4 and 7.

Rhizoctonia solani

Rhizoctonia solani was found in all fields (Table 5). The disease index was highest in field 2 and lowest in field 7.

Table 5. *Damage by* R. solani *presented as averages (median) of all stalks graded in the middle and at the border, respectively. As well as averages (median) of disease index for each field*

Field	Grade Middle	Grade Border	Disease index
1	2	2	2.0
2	4	3	3.3
3	4	3	3.0
4	4	3	3.2
5	4	2	3.0
6	3	2	2.5
7	2.5	2	2.0
8	4	1	2.4

The severity of *R. solani* was greater on plants graded in the middle of the patches compared to those in the margins (p-value 0.02) (Figure 13). The probability of stalks being graded as 4 (completely burnt stalks) was higher than 50 % in the middle while the same probability was less than 25 % at the border.

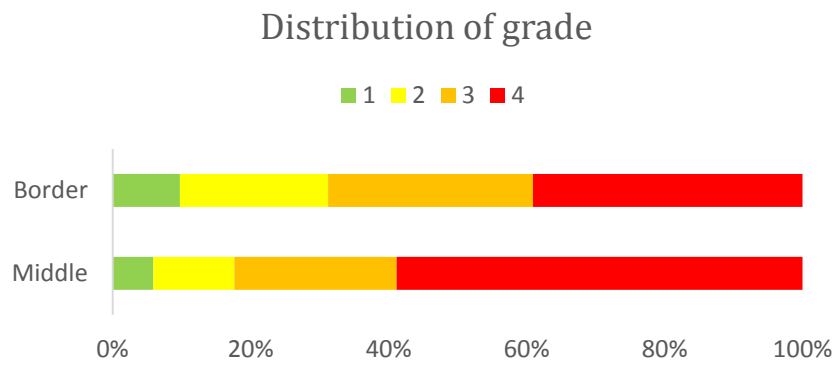

Figure 13. Comparison between plants graded in the middle and at the border of the affected patch, expressed as predicted cumulative probabilities for a certain grade. 1 = weak (one or two very small spots), 2 = defined lesions, 3 = girdled stalks and 4 = completely burnt stalks.

Test of interaction

The interaction between *R. solani* and each nematode group are illustrated in figures 14, 15 and 16. An incline of the curve shows a relationship where higher numbers of nematodes are connected to plants with a higher disease index, in this case more affected by *R. solani*. The interaction between Trichodoridae and *R. solani*, as well as the interaction between *Globodera* spp. and *R. solani*, were both significant. There was no observed correlation between *Pratylenchus* spp. and *R. solani* in this study.

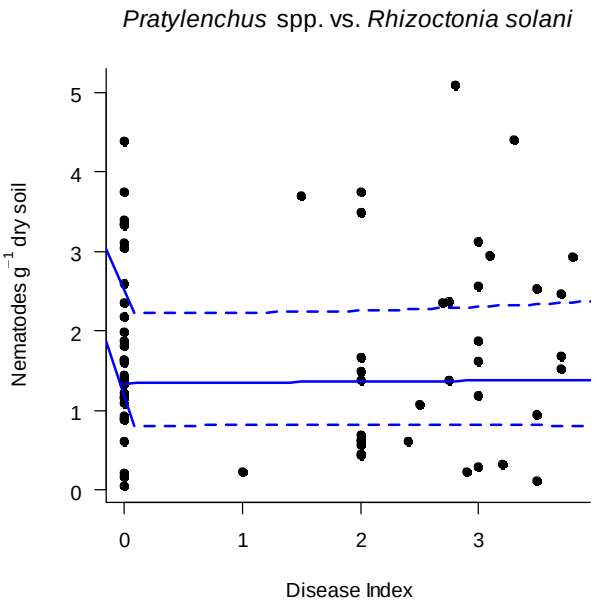

Figure 14. Result of Poisson regression analysis of interaction between *Pratylenchus* spp. and *R. solani*. All eight fields included. The solid line is an estimated regression line with an increase of $e^{(0.008)}$ = 0.8 % per unit DI and the dotted lines show an approximate 95 % confidence interval for the estimated line. DF = 61. The p-value of the interaction is 0.84.

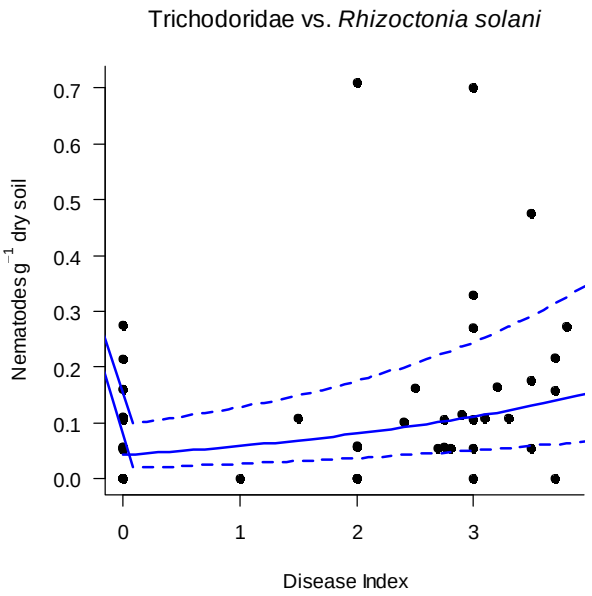

Figure 15. Result of Poisson regression analysis of interaction between Trichodoridae and *R. solani*. All eight fields included. The solid line is an estimated regression line with an increase of $e^{(0.32)}$ = 38 % per unit DI and the dotted lines show an approximate 95 % confidence interval for the estimated line. DF = 61. The p-value of the interaction is < 0.0001.

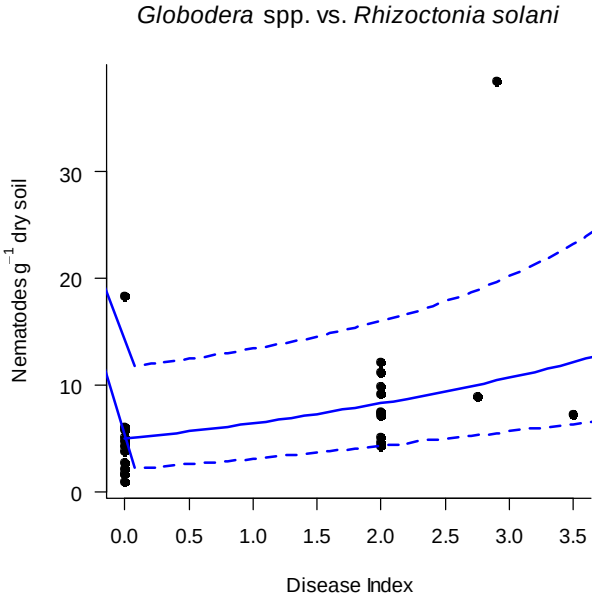

Figure 16. Result of Poisson regression analysis of interaction between *Globodera* spp. and *R. solani*. Field 1, 4 and 7 included. The solid line is an estimated regression line with an increase of $e^{(0.25)}$ = 28 % per unit DI and the dotted lines show an approximate 95 % confidence interval for the estimated line. DF = 21. The p-value of the interaction is 0.03.

The interactions were not significant if excluding the plants which were judged to be healthy by assessment of above-ground symptoms, but were not graded (Table 6). Trichodoridae still had the lowest p-value of the three genus/families tested.

Table 6. *Result of Poisson regression analysis of interaction between* R. solani *and each of* Pratylenchus *spp., Trichodoridae and* Globodera *spp.. All eight fields included but excluding plants not graded. Regression coefficient for the explanatory variable (DI), degrees of freedom (DF), t-value and p-value*

	Regression coefficient for the explanatory variable (DI)	DF	t-value	p-value
Pratylenchus spp.	-0.123	27	-0.84	0.41
Trichodoridae	0.357	27	1.80	0.08
Globodera spp.	0.305	9	0.57	0.58

5 Discussion

I found an interaction between *R. solani* and both Trichodoridae and *Globodera* spp. in the field conditions. Unlike *Globodera* spp. and *R. solani*, the interaction between Trichodoridae and *R. solani* is less studied, and the findings here confirm what advisors and potato farmers in Sweden have suspected. The hypothesis about a spatial correlation is accepted for both Trichodoridae and *Globodera* spp., since a greater severity of stem canker is significantly related to higher numbers of nematodes. The hypothesis is rejected for *Pratylenchus* spp.

As mentioned above, there have not been so many studies of the interaction between Trichodoridae and *R. solani*. Klemmensen (2006) showed similar results on potato in Denmark, otherwise there have been either no interaction (Brodie and Cooper, 1964), interactions between Trichodoridae species and other fungi (Liu and Ayala, 1970) or interaction between another ectoparasitic nematode and fungi (Kisiel et al., 1969). However, the last three studies mentioned have not considered the interaction of these pathogens in potato, but in cotton, sugar cane and corn, respectively.

Potential mechanisms of the synergistic interaction might be limited since trichodorids do not enter the plant with their body. Wounding is restricted to shallow wounds, but they have a relative long stylet and are fast in their ingestion of the cell organelles (< 4 min $cell^{-1}$) (Perry and Curtis, 2013). The question is if these many micropuncture-type wounds are enough to create entry points for the fungus. Evans and Haydock (1993) dismiss that theory. Instead the feeding may slow down the root extension or in other ways affect the root morphology and thereby facilitate the meeting between fungus and plant. Examples of changes in root appearance have been provided by Bowers (1996) and Polychronopoulos (1969), who found *P. penetrans* to stimulate root branching and observed an increase in openings caused by additional emergence of lateral roots when affected by *H. schachtii*, respectively.

Trichodoridae nematodes probably do not change the plant cells in any physiological way but they might inflict some sort of response from the plant that could be beneficial for the colonization of *R. solani*, for example reduced levels of compounds toxic to fungi (Back et al., 2002). Leakage of metabolites into the rhizosphere is probably limited since the nematodes only feed on epidermal cells.

The numbers of Trichodoridae found in the fields were consistently lower, between 6 – 30 times, compared to earlier samplings for free-living nematodes (Andreas Kronhed, Lovang Lantbrukskonsult AB, personal communication). The samples were taken to a depth of 25 cm, this might have been insufficient. Samples for trichodorids are sometimes taken to a depth of 50 cm if, for example, the topsoil layers have dried out (Been and Schomaker, 2013). However, the water content in the soil samples indicated normal water content (6 – 19 %). In temperate zones, only the upper few centimeters are affected by drought and temperature to an extent that would impact the vertical distribution (Been and Schomaker, 2013). The soil samples analysed here were small in comparison to the earlier analyses, and consisted of only one soil core while the other analyses were composite samples from locations all over the fields. The small soil sample increases the risk of missing nematodes which are aggregated in their distribution (Been and Schomaker, 2013). The probability of finding individuals clumped together is higher for Trichodoridae than for *Pratylenchus* spp. and *Globodera* spp.. A single core may therefore be limited as a strategy to sample Trichodoridae, because the sample may not be representative for the population densities outside the core.

Pratylenchus spp. did not show any interaction with *R. solani*. This is consistent with the results from Viketoft et al. (unpubl.) where no difference in the occurrence of stem canker on potato was found when comparing the effects of *R. solani* and *P. penetrans* with the combination of them both. However, in those studies, the tuber yield was decreased when the two of them occurred together, which lead to the conclusion that there indeed was a synergistic interaction between the organisms. I did not investigate the effect on tuber yield in this study. There might have been the same effect, which could be subject for future field studies. Also, there was no discrimination made between different *Pratylenchus* species in this study, and there may have been different results if the analysis had been made for *P. penetrans* only. For example, *P. penetrans*, but not *P. crenatus*, influences colonisation of potato plants by *V. dahliae* (Bowers, 1996), which may be the same for *R. solani*.

Except for the study of Viketoft et al. (unpubl.) there are hardly any records of interaction between *Pratylenchus* spp. and *R. solani* in potato. Regarding other crops, the two pathogens figure together in synergistic interaction on winter wheat (Benedict and Mountain, 1956; Mountain, 1954). Going back to potato, *P. pene-*

trans is also involved in complexes with *V. dahliae* (Bowers, 1996). This complex is dependent on the point of time when the fungus is introduced to the potato plant, since the plant produces hyper-sensitive responses resulting in lignified tissue. This may also explain why *R. solani* did not colonise the potato plants to a greater extent in the vicinity of *Pratylenchus* spp. However, the cultivars tested by Bowers et al. (1996) were not included in this study and there were no antagonistic interaction seen in the analysis. *Pratylenchus* spp. moves inside the root and causes more damage to the roots than Trichodoridae. Logically, that would implicate that *Pratylenchus* spp. would be a better facilitator of entry sites, and movement within the host, for the fungus. However, Bergeson (1972) comments that *Pratylenchus* spp. may destroy the food base for the fungus.

There are also interactions between *Pratylenchus spp.* and other kind of organisms. In Norweigan potato fields, scientists observed more severe symptoms of common scab (caused by the bacteria *Streptomyces scabies*) in areas with high densities of *P. penetrans* (900 specimens $(250\ g)^{-1}$ soil) than in the margins where the growth seemed unaffected (40 specimens $(250\ g)^{-1}$ soil) (Holgado et al., 2009). The researchers concluded that the symptoms easily can be confused with each other, but that the high frequency of scab could be a product of an additive effect. Plant growth was also negatively correlated with the amount of *P. penetrans* in the soil.

The numbers of *Pratylenchus* spp. found in the fields investigated in this study seems reasonable compared to earlier nematode samplings (Andreas Kronhed, Lovang Lantbrukskonsult AB, personal communication).

Globodera spp. were from the beginning not considered, but were included in the study since they were found in such high numbers in three of the sampled fields. In these fields an interaction with *R. solani* was seen. The potato plants in this study were graded 6 – 8 weeks after planting, which is in the same time frame where Back et al. (2006) and Bhattarai et al. (2009) concluded the relationship between *Globodera* spp. and *R. solani* to be strongest. The juveniles counted in this study derived from soil, which means that they had not started forming feeding cells yet. There may, however, have been other individuals in the population which already were inside the roots and affecting the plant. This would be logical since cysts are usually observed on potato roots in the end of June (Nilsson et al., 2012) and the sampling was performed in the middle of June and start of July. The formation of feeding cells creates local physiological changes in the plant (Polychronopoulos et al., 1969), and this might be one reason for the observed interaction with *R. solani*.

Another explanation for the observed interaction between *Globodera* spp. and *R. solani* may be nematode-induced systemic effects. Studies have shown that *G.*

rostochiensis are able to suppress plant disease resistance against fungi through expression of effectors (Postma et al., 2012). Something similar may have made the interaction between *Globodera* spp. and *R. solani* possible.

The cyst nematodes cause necrosis all the way from epidermis to the center of the root when they migrate straight through the cortical cells and the extensive amount of wounding might be the mechanism behind the interaction. Also, leakage of metabolites and changes in their composition might have affected the development of *R. solani* (Back et al., 2010; Dodman and Flentje, 1970; van Gundy et al., 1977). Back et al. (2010) observed the highest growth of *R. solani* on leachates collected 12 days after adding cyst nematodes to the potato plants. This indicates that the modifications by *Globodera* spp. are made early.

It would have been interesting to know whether it was *G. rostochiensis* or *G. pallida* in the samples collected. However, there were no results obtained from the PCR-analyses of potato cyst nematode species. A hypothesis is that the formalin fragmented the DNA, making it impossible to determine whether the sample contained *G. pallida, G. rostochiensis* or even *Globodera* at all (Sigyn Jorde, Intertek ScanBi Diagnostics, personal communication). Medical literature support this hypothesis as well, adding the notion of formalin causing nucleotides to cross-link (reviewed in Srinivasan et al., 2002). Also, formalin-fixed samples should preferably be stored at 4 °C. The samples in this study were stored at room temperature.

There are clearly some disadvantages and obstacles with using DNA based methods when the samples have been stored, instead of using ocular identification under high magnification. In other cases, when the samples have been treated in a way that allows identification through DNA-tests, the method has many advantages. It is fast and precise, and there is no requirement to be a skilled taxonomist (Castillo and Vovlas, 2007; Duarte et al., 2011). Identification through morphological characters is difficult, especially when identifying to species level (Duncan and Moens, 2013; Kumari and Subbotin, 2012).

The cultivars in field 4 and 7 were Folva and Opera, respectively. Both of them are partly resistant to potato cyst nematodes. Folva is resistant against all pathotypes of *G. rostochiensis* while Opera is resistant against only Ro1 and Ro4 of *G. rostochiensis.* The cultivar in field 1 was King Edward, which is susceptible to all potato cyst nematodes. The resistance does not, however, prevent the cyst from hatching, instead it hinders the completion of the nematodes life cycle (Mugniéry and Phillips, 2007). The juveniles counted in this experiment where all extracted from the soil, that is before they entered the root system. Thereby, the potato cultivar does not have any importance on the findings of juveniles.

Instead of the nematodes being the first pathogen leading the way for *R. solani*, it might have been the other way around. For example, the nematodes could have

been attracted to the plants affected by *R. solani* through elevated levels of CO_2 (Edmunds and Mai, 1967, 1966). In less than a month, plant parasitic nematodes can travel 15 cm and occasionally up to 1 m (reviewed in Robinson, 2004). Another possible mechanism is fungal production of cell wall-degrading enzymes which could facilitate entry of the nematodes (Nordmeyer and Sikora, 1983).

It is problematic to set plants that were judged as healthy based on above-ground appearance to a DI 0. The plant may be affected by *R. solani* without showing above-ground symptoms (Åsa Rölin, Hushållningssällskapet, personal communication). It is good that there was a gradient in the damage of *R. solani* within the patch, as that may indicate that there was even less damage outside the patch, as we assumed it to be. The analyses where these non-graded plants were excluded showed no significant interaction. It can either be that the first analysis was biased by the healthy plants or the later analysis did not contain enough sampling spots. The amount of samples analysed were cut from 70 to 36 for the samples containing *Pratylenchus* spp. and Trichodoridae. *Globodera* spp. went from 25 to 13 samples analysed. A limited sample is less likely to give a significant result and it is almost impossible to prove that the sample is normally distributed (Mikael Andersson Franko, Division of Applied Mathematics and Statistics, SLU, personal communication). The use of DI was necessary for the statistical analysis and also transformed a large dataset into a more manageable unit. However, the transformation may have led to losses of data as the graded stalks were merged into one plant unit. DI have been used in other studies of *R. solani* (Atkinson et al., 2010).

There may have been confusion between lesions created by nematodes and cankers inflicted by *R. solani* during the grading. This factor could have been eliminated by bringing the plants into laboratory and confirming the presence of *R. solani* by growing cultures of the fungus. This could have been combined with a more extensive sampling of soil. As mentioned earlier in the discussion, trichodorids are the nematodes most affected by sampling strategy of the nematodes investigated. An improvement would have been to sample a larger volume of soil; perhaps all around the plant, bringing both plant and soil into the laboratory.

Two of the farmers in the study did not use any seed treatment on the tubers, but since the patches of delayed emergence were indeed patches, the conclusion was drawn that it was soil-borne infection. An infection from seed-borne inoculum would have been more randomized in its appearance in the field.

The statistical analysis demanded coordinates in order to handle the distances between the different sampling spots. These coordinates would have been more accurate and easier to provide if each spot of sampling had been recorded with some sort of GPS equipment, instead of estimations and rough calculations.

6 Conclusions

In this study, an interaction in the field between *R. solani* and both Trichodoridae and *Globodera* spp. have been shown. No interaction between *R. solani* and *Pratylenchus* spp. could be found. These results partly confirm what potato producers and advisors have observed and will hopefully give them useful information for future decisions of appropriate management methods. Future work to untangle the mechanisms behind the interactions is needed.

Acknowledgements

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